

# CDK inhibition and cancer therapy

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The cell-division cycle is a tightly controlled process that is regulated by the cyclin/CDK family of protein kinase complexes. Stringent control of this process is essential to ensure that DNA synthesis and subsequent mitotic division are accurately and coordinately executed. There is now strong evidence that CDKs, their regulators, and substrates are the targets of genetic alteration in many human cancers. As a result of this, the CDKs have been targeted for drug discovery and a number of small molecule inhibitors of CDKs have been identified.

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## Abbreviations

<b>CDK</b>	cyclin-dependent kinase
<b>CKI</b>	CDK inhibitory protein
<b>DMAP</b>	6-dimethyl aminopurine
<b>EGF</b>	epidermal growth factor
<b>NGF</b>	nerve growth factor
<b>RB</b>	retinoblastoma

## Introduction

Mammalian cell division is regulated by the timely and coordinated activation of the cyclin-dependent kinase (CDK) family. Regulation of CDK activity occurs at multiple levels, including cyclin synthesis and degradation; activating and inactivating phosphorylation events; CDK inhibitor protein synthesis, binding and degradation; and subcellular localization ([1–5]; see Figure 1). Undoubtedly with the magnitude of research directed at CDKs, further insights into novel mechanisms of their regulation will be revealed. Regulation of CDK activity is essential to the ordered execution of the processes that govern cell growth, complete DNA replication and mitotic transfer of the genome to new daughter cells. To ensure this, surveillance mechanisms function as checkpoints to control cell-cycle progression in case the conditions for advancement have not been met [6,8,9\*,10]. As one of their functions, these signaling pathways exert their effects on cell-cycle progression through CDK regulation. Similarly, as part of their function, growth-promoting signal transduction pathways must transmit their effects on cell-cycle progression by modulating CDK activity [11–14]. As with components of these signal transduction pathways that are so often genetically altered in human cancers, it is befitting that CDKs, their regulators, and substrates, are also frequently the targets of genetic lesions, and promote neoplastic transformation [15,16]. The best-characterized case of such alteration is the retinoblastoma (RB) pathway. Under

normal conditions, phosphorylation of pRb by the Cdk4 or Cdk6 enzyme in complex with one of the D-type cyclins are required for G<sub>1</sub>→S phase transition. Conversely, pRb's unphosphorylated state is essential for mitotic division cycle exit. Cdk4 and Cdk6 are specifically inhibited by the INK4 small molecular weight CDK inhibitor family. It is noted that alterations in one or another component of this pathway is found in nearly all human cancers [15–17].

Excellent reviews have recently documented the multiple modes of CDK regulation, interactions between CDK regulatory pathways and checkpoint control mechanisms and oncogenic alterations of cell-cycle components. Our attempt here is to illustrate the potential for development of therapeutics to treat human cancers by interfering with cell-cycle progression. Because of the central role that they play in advancing the division cycle, CDKs have been targeted for drug discovery and a number of small molecule compounds have now been identified as CDK inhibitors. These strategies and other targets of intervention within the cell cycle are discussed in our review.

## Approaches to CDK inhibition

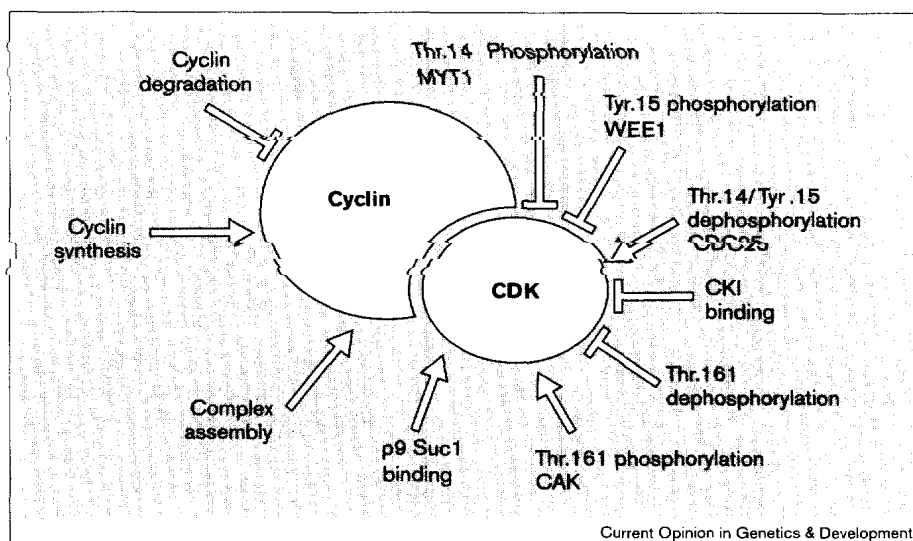
Because of the complex nature of its regulation, modulating CDK activity can be approached via multiple modes for therapeutic intervention. Two basic schemes to inhibit cyclin-dependent kinases are to either directly block the catalytic activity of the CDK, or to target the major regulators of their activity. The most extensively examined of these is catalytic inhibition, which has produced both chemical and peptide/protein based CDK inhibitors. Regulators of CDK activity amenable to therapeutics can encompass: factors involved in the expression and synthesis of the CDK/cyclin subunits or CDK inhibitory proteins, CKIs; proteins that regulate the phosphorylation state of CDKs such as CAK, Cdc25 phosphatases, and the Wee1 and Myt1 kinases; and the machinery involved in proteolytic degradation of the CDK/cyclin complexes or their regulators.

## Chemical inhibitors of CDKs

There are six classes of CDK inhibitors that have thus far been characterized: the purine-based compound olomoucine and its analogues, butyrolactone, flavopiridol, staurosporine and the related compound UCN-01, suramin and 9-hydroxyellipticine. Each is either a natural product or derivative of one with a distinct chemical structure. All occupy the ATP-binding pocket of the enzyme and are competitive with ATP. When examining inhibitors that bind to the catalytic site, especially the catalytic site of an enzyme belonging to a large family such as kinases, the issue of specificity becomes a major issue. However, recent experience and success with the development of effective and specific ATP-competitive inhibitors of a number of

**Figure 1**

Multiple modes of CDK regulation. Regulation of CDK activity occurs at multiple levels, as outlined here. (Thr, threonine; Tyr, tyrosine.) The cdc2 enzyme is used as a reference for sites of phosphorylation (i.e. Thr.14, Tyr.15 and Thr.161). With regard to phosphorylation, the name of the enzyme responsible for a phosphorylation event is given below the event described, for example, threonine 14 phosphorylation is carried out by the MYT1 enzyme.



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kinase enzymes has shown that this task is achievable. Olomoucine and its analogues, butyrolactone, and flavopiridol all show strong specificity for CDKs versus a number of unrelated kinases (see Figure 2 for their chemical structures). Staurosporine, UCN-01 and suramin, on the other hand, show no specificity between the CDKs and other kinases such as PKC [18]. In some cases, such as for 9-hydroxyellipticine the inhibitory activity against kinases other than Cdk2 and Cdc2 is unknown [19,20]. It is interesting to note that of these compounds both olomoucine and butyrolactone inhibit Cdc2 and closely related kinases but do not affect the cyclin-D-dependent kinases Cdk4 and Cdk6. Flavopiridol, on the other hand, can inhibit all CDKs tested including Cdk4 [18]. In collaboration with Parke-Davis Pharmaceutical Research, we have recently identified a chemical inhibitor of the Cdk4 and Cdk6 enzymes by high-throughput screening of a large compound library. This ATP-competitive inhibitor is the first to demonstrate great specificity towards these enzymes versus other CDKs and unrelated kinases (MD Garrett, A Fattaey, unpublished data). In the interests of space, we only discuss further the three classes of CDK inhibitors that show strong specificity for CDKs versus other kinases.

#### *Olomoucine, roscovitine, CVT-313 and purvalanol*

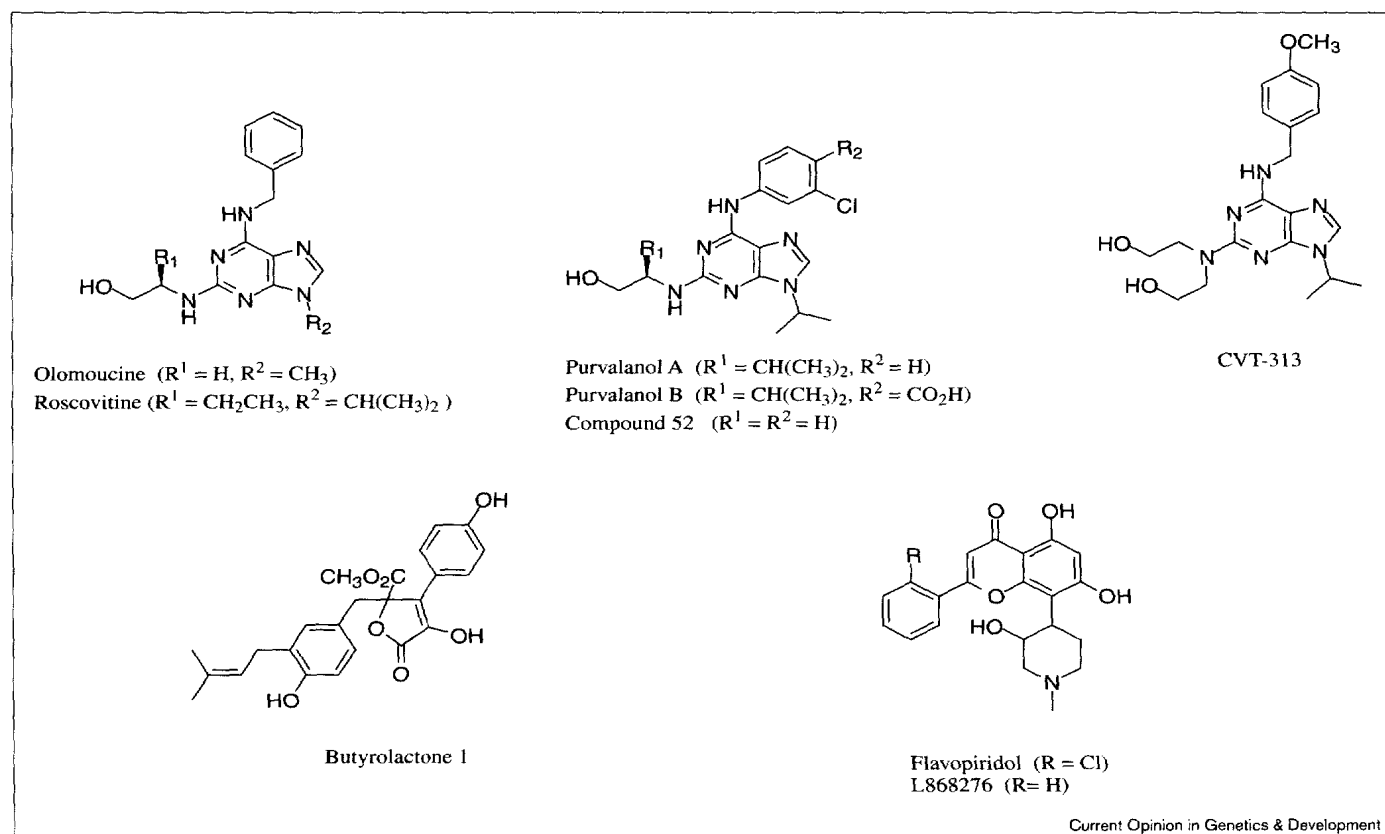
The first compound identified as a Cdc2 inhibitor was 6-dimethyl aminopurine (DMAP; IC<sub>50</sub> = 120  $\mu$ M). DMAP was originally synthesized as a puromycin analogue that blocked mitosis in sea urchin embryos without inhibiting protein synthesis [21–23]. Structural analogy searches identified isopentyl adenine as a slightly more potent DMAP analogue with an IC<sub>50</sub> of 55  $\mu$ M [24]. This strategy led to the discovery of olomoucine as a potent Cdc2 inhibitor (IC<sub>50</sub> = 7  $\mu$ M) with specificity for a subset of CDKs [25]. Among 35 kinases tested, olomoucine only inhibited

Cdc2, Cdk2, Cdk5 and MAP kinase in the micromolar range and did not affect Cdk4 or Cdk6 [25]. Crystallization of olomoucine and a related but weaker inhibitor, isopentyl adenine, with Cdk2 revealed that, although both bound in the ATP-binding pocket of the CDK, the adenine side-chain of each lay in a completely different orientation from the adenine group of ATP [26]. Furthermore, the N6 substituent of olomoucine bound outside the conserved region of the binding pocket making contacts with the protein that are not possible for ATP, suggesting that this interaction is most likely responsible for the specificity of olomoucine towards CDKs.

Another substituted purine compound, roscovitine, is a 10-fold more potent Cdc2 inhibitor compared to olomoucine [27,28\*]. As evident from its crystal structure with Cdk2, roscovitine binds in a similar orientation as olomoucine, with the N6 substituent also making contacts outside the conserved binding domain affirming the likelihood that this interaction provides the specificity of this compound class for CDKs [28\*]. A related compound, CVT-313, has similar inhibitory activity against CDKs as roscovitine and will block neointimal formation in a rat carotid artery restenosis model [29\*].

The most recent addition to these purine-based structures is a group of compounds that were identified in a screen of trisubstituted purine combinatorial libraries designed for Cdk2 inhibition [30\*\*]. The best of these *in vitro* is purvalanol B which shows a thousand-fold increase in potency against Cdk2/Cyclin A when compared to olomoucine. Less potent but more membrane-permeable are purvalanol A (IC<sub>50</sub> Cdk2/cyclin A = 70 nM) and compound 52. Crystallographic analysis of purvalanol B with Cdk2 reveals that binding into the ATP-binding pocket resembles that of the other substituted purines olomoucine and

Figure 2



Chemical inhibitors of CDKs. The structures shown in this figure are of those compounds which specifically inhibit the CDK family of kinases. It should be noted that olomoucine, roscovitine, purvalanol A, purvalanol B, compound 52 and CVT-313 are all trisubstituted purines and thus structurally related.

roscovitine, including the orientation of the N6 substituent away from the conserved region of the pocket, providing specificity towards the CDKs [30\*\*].

Both olomoucine and roscovitine can arrest a number of different cell types in the  $G_1$  and  $G_2$  phases of the cell cycle and block known CDK-dependent cellular activities [31,32\*]. For olomoucine, these include phosphorylation of the Cdc2 substrates protein phosphatase 1 and elongation factors  $\alpha$  and  $\gamma$ , and disassembly of the mitotic Golgi apparatus, whereas roscovitine will block phosphorylation of the Cdc2/cyclin B substrate vimentin [31,32\*,33,34]. In a similar fashion, CVT-313 can block phosphorylation of pRb, a known CDK substrate, and arrest progression through the cell cycle at the  $G_1/S$  and  $G_2/M$  boundaries [29\*]. No cell-cycle data is available for purvalanol A or B. Comparison of the complete mRNA expression profile analysis of a yeast cdc28 mutant with that of wild-type cells treated with compound 52 or flavopiridol revealed the modulation of a common set of genes' expression, suggesting that in this system, both drugs may be inhibiting the cdc28 kinase. Taken together, these data provide sufficient evidence that this class of compounds can inhibit multiple CDKs in the cell. There are also data now emerging that at

least olomoucine and roscovitine can inhibit the growth of cancer cell lines [31,32\*,35,36].

#### Butyrolactone

Butyrolactone was originally identified as a new type of metabolite from *Aspergillus terreus* var. *africanus* IFO8835. It was independently re-isolated from cultured medium of micro-organisms screened for inhibitors of Cdc2/cyclinB [37]. As with the DMAP-derived class of compounds, butyrolactone can inhibit Cdc2 and Cdk2, but not Cdk4, and blocks pRb phosphorylation and entry into S phase in the human WI-38 cell line [18,38]. Butyrolactone also blocks histone phosphorylation and progression through  $G_2/M$  in synchronized WI-38 cells. Cellular effects of butyrolactone can be augmented if cells are treated with digitonin, indicating its poor permeability [39]. Its anti-tumor effects against several lung cancer cell lines *in vitro*, however, have now been well documented [39–41].

#### Flavopiridol (L86–8275)

Flavopiridol is a synthetic analogue of a natural alkaloid isolated from the stem bark of *Dysoxylum binectariferum*, a plant found in India [42]. It was first identified in a tandem screening of the EGF receptor tyrosine kinase inhibition

(IC<sub>50</sub> = 21  $\mu$ M) combined with analysis of inherent cellular cytotoxicity but it was shown to have a far more potent activity against a number of CDKs, including Cdk4. The crystal structure of the related compound L86-8276 (deschloro-flavopiridol) with Cdk2 has revealed that whereas the aromatic portion of the inhibitor binds into the ATP-binding pocket of Cdk2, the phenyl group makes contacts outside of the pocket [43]. This latter association is in a similar manner to the N6 substituent on olomoucine, roscovitine and purvalanol B. This result further strengthens the concept that association of part of a chemical structure with this region of the Cdk2 molecule is important for its specificity towards the CDKs.

As with the purine-based CDK inhibitors, flavopiridol can produce a block in cell-cycle progression at G<sub>1</sub> and G<sub>2</sub>, when added to an asynchronous population of cells [42]. In the breast carcinoma line MCF7, addition of flavopiridol leads to loss of pRb phosphorylation and G<sub>1</sub> arrest [44]. Surprisingly, in these studies, Cdk4 activity is elevated up to three hours after compound addition but has completely disappeared 12 hours post treatment. This lack of kinase activity correlates with the disappearance of cyclin D suggesting that inhibition may be caused by removal of the cyclin partner and not to direct inhibition of the catalytic activity of the enzyme. Flavopiridol has clearly been shown to have growth-inhibitory activity against a number of tumor types *in vitro* and *in vivo* [45–48]. Flavopiridol is now in clinical trials as an anti-cancer drug [49,50].

### CDK inhibitory proteins

Since their discovery, CKIs have frequently been used to investigate the effects of CDK inhibition on tumor-cell growth. Combined with adenoviral vectors as a vehicle for delivery and expression, this is a powerful approach for examining therapeutic applications of CDK inhibition. Adenoviral-mediated expression of CKIs was first described in a report showing that introduction of p16 into lung cancer lines either deleted or, with no detectable expression of the gene, blocked entry into S phase and caused growth inhibition [51]. Tumor studies using these virally infected lines *in vivo* also displayed growth inhibition when p16 was expressed. Since then, a number of similar studies in a variety of tumor types have also demonstrated growth inhibition both *in vitro* and *in vivo*.

Analogous to transfection studies carried out with p16, adenovirus-mediated p16 expression causes growth inhibition in cells with a functional RB gene product but not in tumor lines where it is deleted or mutated [52–55]. Interestingly, none of these studies reported significant apoptosis upon p16 expression; however, co-infection of p16- and p53-expressing adenoviruses could induce significant apoptosis in tumor lines [56]. Studies with adenoviruses expressing either p21 or p27 in cancer cell lines have also demonstrated both *in vitro* and *in vivo* growth inhibition [53,57–63]. Interestingly, two studies with a recombinant adenovirus expressing p27 have report-

ed significant apoptosis in a number of cancer lines *in vitro* [64,65] but it is not proven that this effect is caused by CDK inhibition. In conclusion, gene therapy for cancer using adenoviruses to express p16, p21 or p27 has provided promising results in preclinical studies and could be the basis of a new strategy for cancer gene therapy.

### Peptide-based inhibitors of CDK activity

Peptides that mimic the CDK-inhibitory activity of either p16 or p21 have proven useful as a tool in understanding the changes in cell growth and phenotype caused by these inhibitors. This novel approach combines the power of CDK inhibition with the transmembrane carrier function of a 16 amino acid region of the *Drosophila* Antennapedia protein to deliver the inhibitor into live cells. Treatment of cells with a hybrid peptide corresponding to the third ankyrin repeat of p16, which can bind to and inhibit both Cdk4 and Cdk6 fused to the Antennapedia carrier sequence induced an RB-dependent G<sub>1</sub> cell-cycle arrest and cellular characteristics associated with senescence [66,67,68\*\*]. Full-length recombinant p16 attached to the same 16 amino acid Antennapedia-carrier sequence had similar cellular effects suggesting that, as with adenovirus mediated p16 expression, reconstitution of p16-mediated Cdk4/6 inhibitory activity in cancer cells causes growth inhibition and senescence [69].

Synthetic peptides corresponding to the p21 protein sequence have also been generated but with differing cellular effects. One peptide corresponding to the carboxy-terminal region of p21 (amino acids 141–160) had a 40-fold higher specificity for Cdk4/cyclinD versus Cdk2/cyclinE and gave a potent G<sub>1</sub> arrest in breast cancer lines [70]. Two other p21 peptides generated against amino-terminal regions of p21 (amino acids 17–33 and 63–77) inhibited both Cdk2 and Cdc2 *in vitro* and gave a general blockade in all phases of the cell cycle when introduced into cells [71]. Finally, a number of 20 amino acid peptide aptamers have been identified from a combinatorial library that bind to and inhibit Cdk2 kinase activity in the nanomolar range *in vitro*. At least one of these aptamers appears to do this by blocking the interaction of Cdk2 with its protein partners or substrates [72]. In the future, this technology could be used to identify aptamers that specifically inhibit a variety of protein targets. These approaches suggest that peptidomimetics of CKIs or peptides that inhibit CDK activity could provide novel templates for the development of anti-cancer drugs.

### CDK inhibition: growth arrest, senescence and apoptosis

There are a number of ways to study the role of CDKs in the cell, the simplest being to assess the cellular response to CDK inhibition. In yeast, the power of genetics and the regulation of cell cycle by a single CDK enzyme has been invaluable in understanding the consequences of loss of CDK activity. In human cells, however, the complexity of the CDK family and the programmed suicide system (apoptosis) triggered in response to growth and cell-cycle

perturbations, combined with the multitude of the different cell types that the studies are conducted in, makes the task of understanding the consequences of CDK inhibition rather complicated and confusing. Nonetheless, important knowledge can be gained to understand the implications of CDK inhibition for therapeutic purposes. Cell-cycle arrest followed by either cell differentiation or induction of apoptosis are visibly the most common phenotypes encountered upon inhibition of CDKs in human cells. Van den Heuvel and Harlow [73] first described the use of dominant negative forms of CDKs (DN-CDKs) which showed that overexpression of DN-Cdc2 alleles could specifically arrest cells at the  $G_2/M$  transition whereas expression of DN-Cdk2 and Cdk3 resulted in  $G_1$  arrest [73]. It has been shown more recently that DN-Cdc2, Cdk2 and Cdk3 will suppress apoptosis in HeLa cells induced by staurosporine and TNF $\alpha$  whereas DN-Cdk2 but not DN-Cdc2 will block apoptosis induced by forced overexpression of topoisomerase II  $\alpha$  [74,75]. In contrast, DN-Cdk4 and Cdk6 but not DN-Cdk2 or Cdk3 will protect cultured neurons against DNA damage or NGF deprivation induced cell death, suggesting that different CDKs may play a role in apoptosis depending on the cell type [76]. Heterologous expression of p16 and p21 or peptidomimetics derived from these CKIs also induce  $G_1$  arrest and, in some cases, a senescent phenotype but no apparent apoptosis (see previous two sections). There have been two reports, however, suggesting that overexpression of p27 leads to apoptosis [64,65].

The chemical inhibitors olomoucine, roscovitine, butyrolactone and flavopiridol all arrest proliferating cells at the  $G_1/S$  and  $G_2/M$  boundaries and induce apoptosis [31,32\*,40,41,77–79]. Olomoucine will also trigger an apoptotic response in cells that have been arrested in  $G_2$  by DNA-damaging agents, whereas flavopiridol can induce apoptosis in non-cycling cells [48,80]. In contrast, all these chemical inhibitors can block apoptosis in neuronal cells [81–83]. Finally, there have been recent reports that cleavage of CKIs p21 and/or p27 by caspases leads to Cdk2 activation when apoptosis is induced in human cells [84–86]. In one case, this phenotype could be partially suppressed by expression of DN-Cdk2. Fas-induced apoptosis in Jurkat cells has been reported to cause cleavage of the CDK inhibitory kinase Wee1 and the Cdc27 protein which is involved in cyclin degradation, providing other routes to CDK activation during apoptosis [87]. This would lead us to the conclusion that there must be several factors that determine whether CDK inhibition leads to induction of apoptosis such as the mechanism of inhibition, the cell type, and whether the cell is in cycle and (in some cases) activation of CDK activity may be part of the apoptotic process itself.

### Summing up and the future for CDK inhibitors

We have outlined the potential therapeutic value of CDK inhibition as a cancer treatment and presented the approaches taken to investigate this possibility. To date, only the chemical inhibitor flavopiridol has reached clinical trials and

it has yet to be proven whether its effects are caused by CDK inhibition. Specificity of these compounds is a key factor and one of the future challenges will be to generate more potent and specific CDK inhibitors. Further crystallographic studies of CDKs with novel chemical inhibitors should provide more insight into this issue. In the meantime, there are a number of pharmaceutical companies with ongoing CDK inhibitor programs and non-chemical approaches to CDK inhibition are being tested that may provide us with a CDK inhibitor as an anti-cancer drug in the not too distant future.

### Future prospects

In line with the prevailing CDK-centric view of the cell-division cycle is the fact that the majority of therapeutics under development that target cell-cycle regulation have thus far been CDK inhibitors. Modulators of CDK activity, however, offer as good or greater potential as targets for intervention. As discussed earlier, a number of CDK-activating enzymes (such as CAK and Cdc25 phosphatases) and inhibitory enzymes (such as Wee1 and Myt1) as well as enzymes involved in modulating their activity, can now be considered.

Although the CAK-dependent CDK activation step appears to be constitutive, inhibitory phosphorylation of CDKs and their activation by Cdc25 phosphatases is highly regulated and is the target of checkpoint surveillance mechanisms [88–90]. This is especially well characterized for the  $G_2$  to M transition and the checkpoint signaling pathway invoked in response to DNA damage where the inhibitory phosphorylation on Cdc2, along with a loss of Cdc2 activity is sustained. Using such agents as caffeine and UCN-01, abrogation of this DNA-damage-induced  $G_2$  checkpoint correlates well with loss of phosphorylation on at least tyrosine 15 of Cdc2 and an increase in Cdc2 activity [91–94]. In addition, exogenous expression of Cdc2 alleles containing non-phosphorylatable amino acids at position 14 and 15 prevents the  $G_2$  arrest induced by ionizing radiation [95–97]. These studies illustrate a significant loss in cell viability, indicating that abrogation of the  $G_2$  checkpoint, especially in p53 null cancer cells, may be a way to target them for cell death.

Another area of CDK/cyclin regulation with therapeutic potential is the control of CDK, cyclin or CKIs levels in the cell. Antisense technologies have recently been applied to eliminate Cdk2 mRNA levels in cells [98–101]. Cyclins and CKIs are regulated at the transcriptional and protein stability level. Much has been learned about the enzyme complexes and the signals involved in targeting the cyclins and to a lesser extent the CKIs for ubiquitin-mediated proteolysis [102,103]. An increase in the proteolysis of specific cyclins may be one way to inactivate the CDKs and conversely inhibition of CKI turnover may yield cell-cycle arrest and cytostasis [104]. The p27 CDK inhibitor may be an attractive choice in this respect, as it is active in mid to late  $G_1$ , and its levels are either reduced or absent in a number of malignancies [105–109]. Decreased levels of

p27 are also indicative of poor prognosis in young breast cancer patients [110]. Most recently the murine p27Kip1 gene has been shown to be haploinsufficient for tumor suppression, which establishes a causal link between loss of p27 and a predisposition to tumors [111]. It can therefore be safely said that as our understanding of the cell cycle and its role in tumor progression expands, new approaches and targets for intervention and treatment of human cancers must surely be just around the corner.

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